

THE CORRELATION OF VARIATION IN DYE
SENSITIVITY WITH GRAM CHARACTER
IN CERTAIN GRAM-POSITIVE
ORGANISMS

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ESTHER WAGNER STEARN, A.B., M.S.

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PARTIAL FULFILLMENT OF THE REQUIRE-
MENTS FOR THE DEGREE OF DOCTOR
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THE CORRELATION OF VARIATION IN DYE SENSITIVITY WITH GRAM CHARACTER IN CERTAIN GRAM-POSITIVE ORGANISMS¹

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The researches reported in this paper were stimulated by the anomalous behavior met with on the part of certain Gram-positive organisms during the course of a series of investigations on the mechanism of dye bacteriostasis. The data presented were assembled to determine whether variation in Gram character is accompanied by, and thus implies, corresponding variation in isoelectric range, and also corresponding variation in resistance to gentian violet when used in nutrient media, or not.

In an investigation previously reported (Stearn and Stearn, 1926) it was found that the static action of dyes on bacteria could be explained on the assumption of the formation of an equilibrated dye-bacterial compound which behaved as one would expect an "ionic" or "salt" type of compound to behave. A basic dye was more effective as alkalinity was increased while the opposite was true for acid dyes. Moreover the equilibrated condition was demonstrated by showing the reversibility of the static action. This was demonstrated by culturing under conditions of dye concentration and pH which just inhibited growth of the organism for ninety-six hours, and then, in case of a basic dye, merely decreasing the pH, keeping all other factors constant, and remaining always within the pH range of growth for the particular organism being studied. After such a pH shift growth was obtained.

In all cases of Gram-negative organisms studied perfectly

¹ The abstract of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Missouri.

regular results were obtained without exception. In several cases, with Gram-positive organisms, unexpected results were obtained. These are shown in table 1 and indicated by the asterisks. This table gives results of incubation after inoculation with the indicated organism into a 0.2 per cent lactose broth, and adjustment to the pH indicated by means of phosphate buffers.

TABLE 1

	GENTIAN VIOLET DILUTION	pH 5.9	pH 6.9	pH 7.1	
<i>Streptococcus hemolyticus</i> (limiting pH range 5.5 to 8.0)	4,000,000	+	—	—	72-hour results
	3,000,000	—	—	—	
	2,000,000	+*	—	—	
	1,000,000	—	—	—	
		pH 6.0	pH 7.0	pH 7.6	
<i>Staphylococcus pyogenes</i> <i>aureus</i> (limiting pH range 5.6 to 8.1)	3,000,000	+	—	—	72-hour results
	2,000,000	—	—	—	
	1,000,000	+*	—	—	
		pH 5.2	pH 6.2	pH 7.2	
<i>Bacillus cereus</i>	4,000,000	+	+	—	24-hour results
	2,000,000	—	+*	—	
	1,000,000	—	—	—	
		pH 6.0	pH 6.6	pH 7.0	
<i>Bacillus subtilis</i> (limiting pH range 4.5 to 8.5)	4,000,000	—	—	—	48-hour results
	3,000,000	—	—	—	
	2,000,000	—	+*	—	

The media contained varying concentrations of gentian violet as shown.

It will be noted that in the cases indicated by the asterisk growth was obtained in an environment in which, from the general behavior shown by the table, one would not expect it. Examination of the cultures from each of the tubes exhibiting the unexpected behavior revealed the presence of both Gram-negative and Gram-positive organisms, in place of the purely positive strains with which the work had been started. Smears made

from subcultures taken from these tubes gave the same mixed picture.

The present communication embodies the report of a study of these variants. The scope of the study may be briefly outlined as follows:

1. Attempts to obtain pure Gram-negative strains (*a*) by mechanical selection, (*b*) by continued subculturing in acid and alkaline media.
2. Investigation of the persistence of the change in Gram character.
3. Investigation of the accompanying change in dye sensitivity.
4. Investigation of the accompanying change in isoelectric range.

EXPERIMENTAL

Extensive work was confined to those cultures of *Staphylococcus pyogenes-aureus*, *Bacillus cereus* and *Bacillus subtilis* obtained from the tubes whose cultures had so definitely shown a tendency to vary from the usual reaction. The *Staphylococcus pyogenes-aureus* came from the infected lip of a patient in the Pasadena Hospital. The cultures of *Bacillus cereus* and *Bacillus subtilis* came from the Natural History Museum, Washington, D. C.

A strain of *Streptococcus hemolyticus* was isolated in pure culture from the spinal fluid of a meningitis patient in the Pasadena Hospital. The work with the variant from this strain showed a behavior paralleling that of the other strains as far as it was carried.

1. Attempts to produce a pure negative strain

The general procedure consisted in streaking the culture from acid and alkaline broth, the pH of which was adjusted by means of phosphate buffers, to plain agar. After twenty-four hours incubation, colonies were examined by means of fixed smears, and stained by the classic Gram technique. The colonies showing the greatest preponderance of Gram-negative organisms were transferred to 0.2 per cent lactose broth adjusted to a pH of 5.2, and those showing a preponderance of Gram-positive organisms were transferred to broth adjusted to a pH of 7.8 to 8.0. These

resulting cultures were again streaked to plain neutral agar and the same process of mechanical selection and subculturing into acid and alkaline media repeated.

The results obtained from the daily examination of a large number of the isolated colonies on twenty-four-hour plates, streaked from the variant of *Staphylococcus pyogenes-aureus*, are given in table 2.

TABLE 2

DAY	NUMBER OF COLONIES EXAMINED	NUMBER OF COLONIES MIXED	NUMBER OF COLONIES MOSTLY BLUE	NUMBER OF COLONIES MOSTLY RED
1	26	19	3	4
2	20	14	6	—
3	25	22	3	—
4	26	24	2	—
5	26	24	2	—
6	25	23	—	2
7	22	16	3	3
8	39	28	10	1
9	20	15	5	—
10	43	29	6	8
11	54	34	20	—
12	77	70	4	3
13	67	61	1	5
14	72	58	3	11
15	73	61	4	8
16	75	71	1	3
17	15	15	—	—
Totals.....	705	584	73	48

The part of the colony taken for making the smear did not seem to affect the relative number of red and blue cocci.

In general, pure mechanical selection did not seem to increase significantly the preponderance of Gram-negative organisms.

Incubating in media of a comparatively low pH for long periods of time did, however, in case of all three organisms give a product which, though still mixed, was not more than 50 per cent Gram-positive. No cases of pure negative smears were noted.

The change in staining character, as a statistical change, is extremely slow. All three of the organisms studied gave smears

of the original variant which were preponderantly Gram-positive. It was only after months of subculturing at a low pH that a strain statistically equal in positive and negative individuals could be obtained. The change is a reversible one, but it is significant to note that reversion is as slow and as difficult of accomplishment as the original change. The strain of *Bacillus cereus*, after it had been cultured for months at a low pH and was yielding smears with about the same number of negative as of positive organisms, was then cultured in broth at a higher pH. Even after three months of such culturing smears still showed Gram-negative organisms, though finally to an extent much less than when the culturing at the higher pH was begun.

It should be stated that on the same slide with the smears of variant under examination there was also placed a smear of the original purely Gram-positive organism which had been maintained at a high pH. This insured identical treatment of the two smears in the staining technique and permitted comparison of the variant with the orthodox strain. This latter retained its pure Gram-positive character throughout the course of the work.

The slowness of the reversion of the variant, as well as the fact of mixed strains, seems to the authors to constitute fairly definite proof that the Gram reaction of an organism does not depend exclusively, or even primarily, on the surface condition of the organism, but is due to characteristics more fundamental. These have been fully discussed previously (Stearn and Stearn, 1924 a and b).

To meet possible objections that mixed smears might be due to mixed or impure cultures, a large number of single cell cultures were obtained, using a modification of Orskov's method (1922). These cultures, in all cases showed the same mixed character as did the cultures from which they were obtained. It should also be remarked that a control culture of the Gram-negative organism, *Bacillus coli*, was "carried along" through similar procedures for long periods of time, but in no case was any change in Gram character noted. In no cases was a mixed smear obtained even after long culturing at a high pH.

TABLE 3
Bacillus cereus

DILUTION OF GENTIAN VIOLET	VARIANT, SHOWING ABOUT 50 PER CENT GRAM-NEGATIVE RODS			ORIGINAL STRAIN SHOWING A PURE GRAM-POSITIVE SMEAR		
	pH 5.2	pH 6.2	pH 7.7	pH 5.2	pH 6.2	pH 7.7
1,000,000 {	+	-	-	-	-	-
	+	-	-	-	-	-
	+	+	-	-	-	-
2,000,000 {	+	-	-	-	-	-
	+	-	-	-	-	-
	+	+	-	-	-	-
4,000,000 {	+	+	-	-	-	-
	+	+	-	+	+	-
	+	+	-	+	+	-
6,000,000 {	+	+	-	+	+	-
	+	+	+	+	+	-
	+	+	+	+	+	+

Results are given for twenty-four, forty-eight and seventy-two hours incubation.

TABLE 4
Bacillus subtilis

DILUTION OF GENTIAN VIOLET	VARIANT, SHOWING ABOUT 50 PER CENT GRAM-NEGATIVE RODS			ORIGINAL STRAIN SHOWING A PURE GRAM-POSITIVE SMEAR		
	pH 5.2	pH 6.2	pH 7.7	pH 5.2	pH 6.2	pH 7.7
1,000,000 {	-	-	-	-	-	-
	-	-	-	-	-	-
	-	-	-	-	-	-
2,000,000 {	+	+	-	-	-	-
	+	+	-	-	-	-
	+	+	-	-	-	-
4,000,000 {	+	+	+	-	-	-
	+	+	+	+	+	-
	+	+	+	+	+	-
6,000,000 {	+	+	+	+	+	-
	+	+	+	+	+	+
	+	+	+	+	+	+

Results are given for twenty-four, forty-eight and seventy-two hours incubation.

2. Variation in sensitivity to dyes

Two distinct series of experiments were carried out. The first was by means of direct transfer from colonies on agar showing a tendency toward Gram-negativeness, and the other was by the transfer of two drops of a twenty-four-hour acid and alkaline broth culture, each into sterile distilled water. These tubes were thoroughly shaken and allowed to stand for ten minutes after which one drop was inoculated into 0.5 per cent lactose gentian violet broth. The variant which had been cultured at low pH and that which had been cultured at high pH were always run parallel, using identical media.

TABLE 5
Staphylococcus pyogenes-aureus

DILUTION OF GENTIAN VIOLET	VARIANT, SHOWING ABOUT 50 PER CENT GRAM NEGATIVE RODS			ORIGINAL STRAIN SHOWING A PURE GRAM-POSITIVE SMEAR		
	pH 6.0	pH 7.0	pH 7.7	pH 6.0	pH 7.0	pH 7.7
2,000,000	+	—	—	—	—	—
3,000,000	+	—	—	+	—	—
4,000,000	+	+	+	+	+	—

Results are given for seventy-two hours incubation.

Tables 3 and 4 give the results of one typical experiment on two of the three organisms studied. In both cases the variant came from a single cell culture. Comparison is made with the non-variant strain kept continuously at a high pH. These tables show the decrease in sensitivity to dye paralleling the partial loss of Gram-positiveness. This difference in sensitivity to gentian violet on the part of the two strains of the same organism was repeatedly confirmed.

In the case of the results given in table 5 for *Staphylococcus pyogenes-aureus*, the sensitivity of the variant is compared with that found for the original strain at the beginning of the work. Results are given for seventy-two hours incubation.

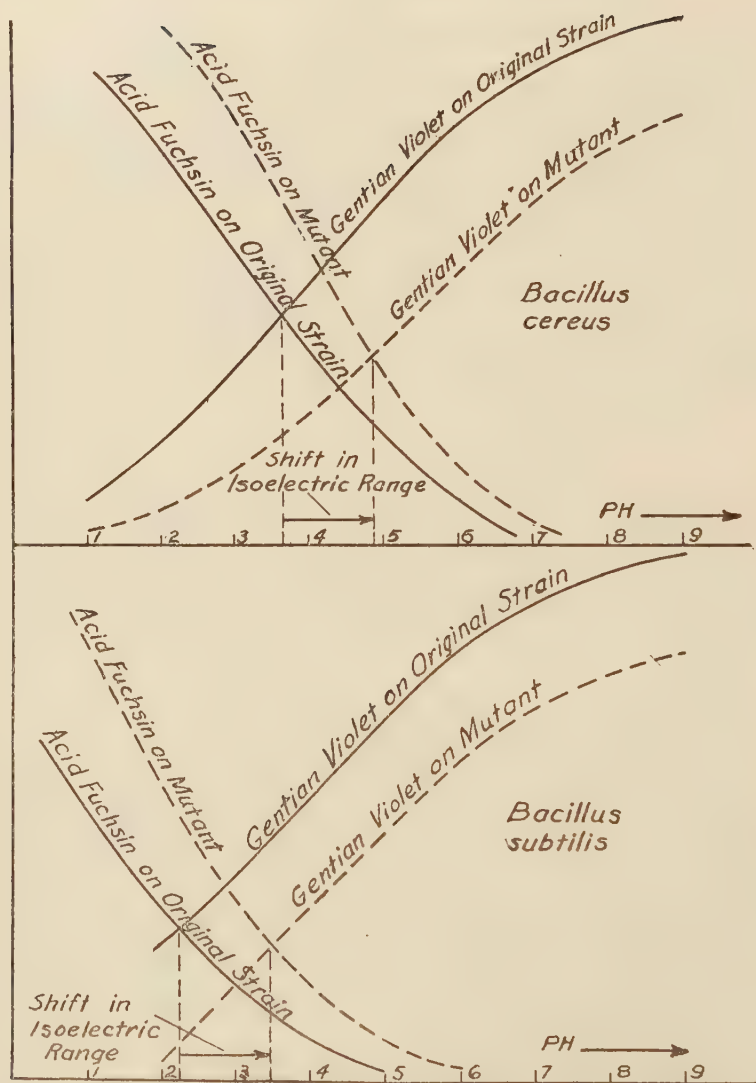


FIG. 1. SHOWING THE SHIFT IN ISOELECTRIC RANGE PARALLELING THE CHANGE IN GRAM CHARACTER AND IN SENSITIVITY TO GENTIAN VIOLET

The broken line gives the behavior of the variant, the continuous line that of the original strain. Abscissae give pH values while ordinates represent intensity of retained color, after decolorizing with acetone, on an arbitrary comparative scale. For the experimental method of determining these curves see Stearn and Stearn, 1924a.

3. Changes in isoelectric range

The character most important in determining whether or not gentian violet is strongly retained by an organism has been shown by Stearn and Stearn (1925) to be the specific acidity of the organism. By this is meant its tendency to combine with basic substances such as gentian violet. The organisms most strongly Gram positive are most acidic in nature. The amphoteric nature of bacteria has also been demonstrated by the authors (Stearn and Stearn, 1923), and it has been shown that the isoelectric range is a measure of the acidic strength of bacteria. The more strongly acidic an organism is, the lower is the pH of the isoelectric range. One might expect, then, with loss of Gram positiveness, a corresponding shift of isoelectric range to a higher pH.

The isoelectric ranges of *Bacillus cereus* and *Bacillus subtilis* were determined for the original strains and for the variants. The results are given graphically in figure 1. In both cases a distinct shift is noted in the isoelectric range toward a higher pH.

DISCUSSION

One of the outstanding questions arising from the experimental work presented in this paper is whether the variation of strains, discovered in the cases of the Gram-positive organisms growing under adverse environmental conditions, is due to the purely chance presence of an already largely adapted variant which will develop more rapidly and prolifically than its less fortunate brothers and thus tend to breed a more resistant strain by selection, or whether, as one approaches a limit in imposing adverse environmental conditions on a growing organism, one reaches a fairly definite point on the one side of which no growth takes place but on the other side of which there is a gradual tendency toward adaptation on the part of the majority of the organisms present. Such a question is not easy to answer and will probably form the basis for considerable future experimentation.

The study of variation by watching the change in Gram character, though interesting and important in itself, will probably appear at first sight as a comparatively trivial matter and one

of limited importance. Such, however, will be by no means the case when it is shown that the Gram reaction need not be confined to an arbitrary classification of organisms, but that, in the case of any individual species, variation in Gram character is paralleled by, and is thus a reflection of, variation in sensitivity to dyes. That Gram-positive organisms are, on the whole, more sensitive to basic dyes than Gram-negative ones is generally known. The experimental evidence presented in this paper goes farther, however, and shows that, in the case of the *same* species of organism, loss in Gram-positiveness is accompanied by decreased sensitiveness to the basic dye gentian violet.

Regardless of the reason for the first appearance of the variant strains, two facts are of note as brought out in this study.

1. After its original appearance, the variant persists even after long subculturing under conditions favorable to the "orthodox" strain.

2. It has been found impossible to obtain a culture in which all of the organisms are individually variant, though attempts to obtain such a culture were made both by mechanical selection and by long continued subculturing under adverse environmental conditions such as at a low pH.

Reference to table 2 shows that, out of over 700 colonies examined, about 83 per cent were mixed colonies in which neither Gram-positive nor Gram-negative organisms predominated to any considerable degree, about 10 per cent were mostly Gram-positive while about 7 per cent gave almost pure smears of the Gram-negative variant.

Regarding the first of the two facts given above, it should be stated, to avoid misconception, that the statistical persistence of the variant strain is definitely dependent on its environmental conditions. Under proper conditions, there is a tendency to an extremely slow reversion to the original strain, i.e., when culturing conditions favorable for the development of the original strain are used. In this work, however, though such a tendency was clearly indicated, complete reversion was never obtained.

Another significant fact brought out in this study is that the formation of these chance variants has occurred, in our experi-

ments, only in the case of Gram-positive organisms. No cases of a chance alteration of a Gram-negative to a Gram-positive organism have been noted. Such a change is fundamentally different, however, from the change—Gram-positive to Gram-negative. In the latter case there is the loss of a character—the retaining power for gentian violet. It is more or less absurd to speak of the loss of negativeness. The change, negative to positive, entails not the loss but the acquisition of a character—the retaining power for gentian violet—and one might well expect chance loss of positiveness to appear much more frequently in the case of a typical Gram-positive organism than the acquisition of positiveness by a typical negative organism. The latter change may possibly be induced, but it would be a matter of forcing the character onto the organism and the methods would be, in general, more rigorous.

Churchman (1921), though he was unable to train *Bacillus subtilis* to grow in the presence of gentian violet, did succeed in obtaining a strain of *Bacterium coli* whose sensitiveness to gentian violet was markedly increased over that of ordinary cultures. This was accomplished, not by special treatment of the original culture, but by a technique of selection in which increasingly dilute suspensions were streaked on divided agar plates until finally growth occurred only on the side of the plate free from gentian violet. The results are explained by Churchman as due to the fact that only a comparatively small proportion of the organisms in any strain of *Bacterium coli* will grow in the presence of the dye, but that with heavy inoculations there are enough of those which will grow to produce satisfactory growth. The “gentian violet positive” strain thus developed was still Gram-negative in reaction, and thus the conclusion was reached that “the factor which determines the reaction of an organism to the Gram process is not the same as the factor which determines its growth in the presence of gentian violet.” No data as to concentration of dye used nor pH of media are given in the report, so that critical interpretation of the results is impossible.

In the attempts to acclimatize *Bacillus subtilis* to gentian violet it was reported that in some of the tubes in which there was a

concentration of dye 1:1,000,000 there were a "few organisms" developed. No report of the Gram character of these "individual cases" is given, though it is possible that had they been examined, it would have been found that they contained both the Gram-positive and Gram-negative organisms in the same way that those seemingly chance "individual cases" reported in this study did, and which in the present case furnished the starting point for the work on the Gram-positive organisms.

If Churchman's explanation of the development of a gentian violet positive strain of *Bacterium coli* is valid, his results furnish some basis for the idea that, in the appearance of these variant strains, we are dealing with variants already present in any culture, and which require only an environment which, though adverse to the culture as a whole, still permits them to multiply so that we have a partially selective growth.

The results, however, certainly do not warrant any definite conclusion that the factor which determines Gram character is not the same as that which determines sensitiveness to gentian violet. The comparatively large amount of systematic work done by the present authors in their various studies in which determining factors were discovered and definitely controlled indicates a close parallel between Gram character and sensitiveness to basic dye. The apparent contradiction to this idea in the work of Churchman reviewed here is one of degree only. Gram-positive organisms may lose the retaining power for gentian violet, and, though their sensitiveness to this dye is noticeably decreased, paralleling this loss, they are even yet more sensitive than ordinary typical Gram-negative strains. The Gram technique is a rather crude and rigorous procedure from the point of view of a live organism, and one might well expect slight increases in retaining power for gentian violet to be sufficient seriously to affect the viability of a growing culture, even though they were so small that, with such vigorous decolorizing as given in the gram technique, no difference could be noted—using such crude methods as visual observation and even clouding this observation with a strong counterstain. Gram negative organisms which counterstain to a brilliant red with the proper dye might, and often would,

appear far from completely decolorized if no counterstain were used; the phenomenon is one of gradation in effect.

From this more general point of view it may well be expected that an organism may become distinctly more sensitive to gentian violet but still be classified as Gram-negative, while another organism may lose its Gram-positiveness but still retain a fairly high sensitiveness to gentian violet.

It should finally be pointed out that the experimental changes brought about in the variants as described in the foregoing paper were *not* brought about by acclimatization methods. The decreased sensitivity to gentian violet on the part of the variants was not due to the acclimatizing effect of culturing for long periods of time in the presence of small amounts of the dye. The shift in isoelectric range, the change in Gram character and the decreased sensitivity to gentian violet are all reflections of some more fundamental change which the organisms have undergone.

SUMMARY

1. Chance variants were discovered in several cases of strongly Gram-positive organisms growing under adverse environmental conditions.

2. This variation has been shown to persist.

3. It has not yet been found possible, either by mechanical selection, or by the modification of growth media either by exaggeration or modification of the adverse conditions, to isolate a pure strain of the variant.

4. Experiments have been adduced to show that, though the original variant seemed to be due to chance, any subsequent change in the strain is very gradual. By long subculturing under a certain constant set of conditions the variant tendency may be greatly increased, while under another constant set of conditions a tendency to an extremely slow reversion to the original strain is noted.

5. Loss of Gram-positiveness, i.e., decrease in affinity for basic dye, has been shown to parallel exactly the decrease in sensitiveness to basic dye.

6. Loss of Gram-positiveness has been shown to be accom-

panied by a definite shift in the isoelectric range of the organism. This shift is in the direction which one would expect from the study of the isoelectric ranges of the typical Gram-positive and Gram-negative organisms.

7. All of the results obtained in this study are completely in accord with, and furnish confirmation of, the general theory of the Gram reaction and of dye bacteriostasis as developed during recent years by the author.

In conclusion the author wishes to acknowledge her indebtedness to Dr. M. P. Ravenel of the Department of Preventive Medicine of the University of Missouri, for the interest he has shown during the course of this work, and for assistance in the preparation of this manuscript.

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BIOGRAPHY

The writer of this thesis was born in Decatur, Illinois. Her early education was received in the public schools of Forest Park and of Maywood, Illinois. After graduating from the Proviso Township High School, of Maywood, Illinois, she spent a year in residence at Lewis Institute, Chicago, Illinois. She entered the University of Illinois in the autumn of 1916 and graduated with the degree of Bachelor of Arts in chemistry in the spring of 1918. In the summer of 1920 she received the degree of Master of Science from the University of Illinois. The thesis consisted of the report of a study of a "Lactose Broth for Isolating Bacterium Coli from Water". (*American Journal of Public Health*, v. 11, 1921, p. 203.) This work was carried out under the direction of Professor W. F. Monfort.

She entered the Graduate School of the University of Missouri in the autumn of 1923 and has pursued graduate studies intermittently since that time. The years 1924 and 1925 were spent in prosecuting researches in the Pasadena Hospital, Pasadena, California.

Since her graduation from the University of Illinois the writer has held the following positions: Assistant Bacteriologist, Illinois State Water Survey, 1918; Bacteriologist and Sanitary Chemist, Illinois State Board of Public Health, 1919; Instructor in Chemistry, University of West Virginia, 1920; Instructor in Chemistry, University of Missouri, 1921 and 1922; Assistant Bacteriologist, Missouri State Public Health Laboratory, 1922; Instructor in Chemistry, Christian College, 1923 to date; Research Bacteriologist, Pasadena (California) Hospital, 1924 and 1925.

During the years since her graduation she has interested herself in:

1. Bacterial Mutation and the Life Cycles of Bacteria;
2. Staining Reactions of Bacteria;
3. Dye Therapy.

Following is the bibliography of titles of publications of which she has been author or co-author to the present time:

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